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BCX-1812 (RWJ-270201): Discovery of a Novel, Highly Potent, Orally Active, and Selective Influenza Neuraminidase Inhibitor through Structure-Based Drug Design

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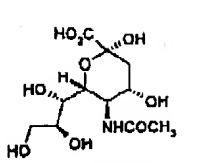
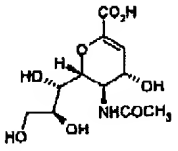
Introduction. Influenza remains the major cause of mortality and morbidity among respiratory diseases.[1,2] Until very recently, there were only two options available to reduce the impact of the influenza virus: (1) vaccines, which although effective are underutilized and not completely protective because of frequent antigenic shift in the viral surface proteins, and (2) the antiviral drugs, amantadine and rimantadine, which are limited in their effectiveness because of the rapid emergence of resistant viral strains and lack of activity against influenza B virus.[3]

Hemagglutinin and neuraminidase (NA), the two glycoproteins on the surface of the influenza virus, have long been considered as potential antiviral targets.[4] Neuraminidase cleaves terminal sialic acid residues from glycoconjugates, promoting the release of newly formed virus particles from infected cells. Studies with a neuraminidase-deficient influenza virus have shown that the mutant virus is still infective but the budding virus particles form aggregates or remain bound to the infected cell surface.[5] Thus, influenza neuraminidase has been considered an attractive target for the development of new drugs for influenza.

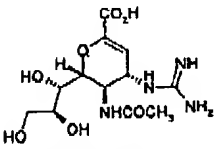
Recently, two influenza neuraminidase inhibitors, zanamivir (**3**, 2,4-dideoxy-2,3-didehydro-4-guanidino-sialic acid; Relenza) from Glaxo Wellcome and Biota and oseltamivir (ethyl 4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate; Tamiflu) from Hoffman La Roche and Gilead Sciences, have been approved by the FDA for the treatment of influenza.[6,7] Zanamivir is administered by oral inhalation, and oseltamivir is a prodrug that is converted to the active form, GS4071 (**4**), upon oral administration. Here, we report the design and synthesis of a new class of potent and selective influenza neuraminidase inhibitors that are structurally different from sialic acid (**1**) and its analogues and are orally active against both influenza A and B viruses.



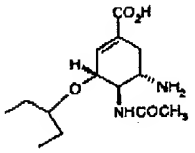
1 (Sialic Acid)



2 (DANA)



3 (Zanamivir)



4 (GS4071)

Inhibitor Design. The sialic acid analogue, 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (DANA, **2**) was reported to be an inhibitor of influenza neuraminidase with an IC_{50} value of 5−10 μM.[8] The crystal structures of influenza A neuraminidase complexed with sialic acid and DANA have been reported previously.[9] The interactions of DANA with the enzyme are characterized by both strong charge−charge interactions and limited hydrophobic contacts. Figure 1 shows the active site consisting of four binding pockets: (1) an acid pocket where the carboxylic acid of DANA has hydrogen bond interactions with a triad of arginine residues (residues 118, 292, and 371), (2) an acetamido binding pocket that has a hydrophobic patch formed by Trp 178 and Ile 222 and a buried water molecule, (3) the glycerol binding pocket, and (4) the fourth pocket into which the C4-hydroxyl of DANA is positioned. A water molecule surrounded by negatively charged residues characterizes the fourth pocket.

Our main objective was to develop novel, orally active, potent, and selective inhibitors of influenza neuraminidase. This led us to explore ring structures that are different from sialic acid and its analogues. We also concentrated on replacing the glycerol side chain, which may interfere with oral bioavailability.[10] α/β-6-Acetylamino-3,6-dideoxy-D-glycero-altro-2-nonulofuranosonic acid (**5**) is known to inhibit influenza neuraminidase with a potency comparable to that of DANA.[11] While it has the same substituents as DANA and sialic acid for interaction with the four binding pockets of the active site, their arrangement on the ring is very different. On the basis of crystallographic studies of DANA and of sialic acid bound to neuraminidase, it has been proposed that DANA mimics the transition state and hence has improved potency over sialic acid.[12] Even though the central ring structure of compound **5** is different from that of DANA, its comparable potency to DANA's led us to investigate the crystal structure of **5** bound to N9 influenza neuraminidase that revealed some interesting and important features. The superposition of NA complexes containing DANA and compound **5** shows that the ring in **5** is significantly displaced from the pyranose ring of DANA (Figure 2). However, the main functional groups (carboxylic acid, glycerol, acetamido group, and C4-hydroxyl group) in both the complexes have the same

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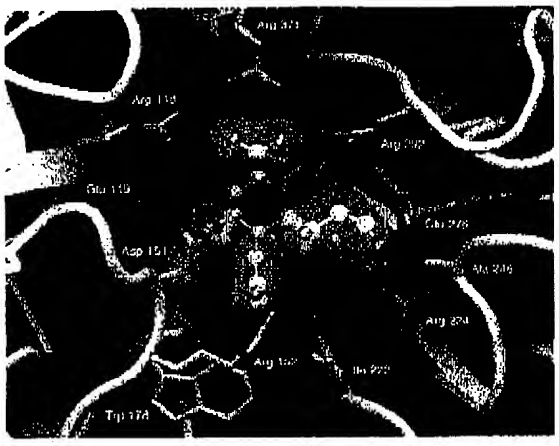


Figure 1. Binding of DANA (2) to influenza A neuraminidase N9 showing the four binding pockets (depicted as transparent surfaces) in the influenza A neuraminidase N9 active site.

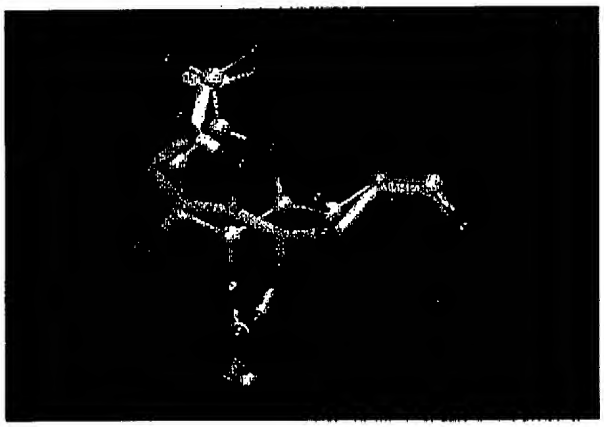
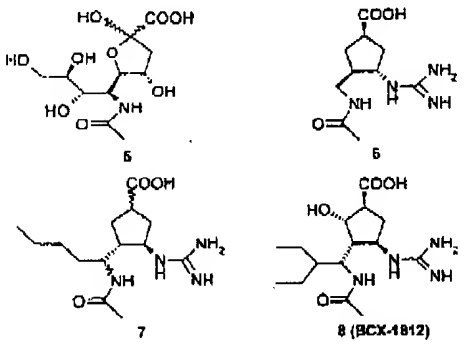


Figure 2. Superposition of compounds 2 (ball-and-stick) and 5 (liquorice) in the conformations of the molecules when bound to influenza A neuraminidase N9.

relative positions in the active site and have similar interactions with the enzyme. This crystal structure demonstrates that what is important for potent neuraminidase inhibition is not the absolute position of the central ring but rather the relative positions of the interacting groups. This is a critical point in an active site that is predominantly charged such as NA, since electrostatic interactions are highly dependent on the relative positions of the interacting groups.



Cyclopentane Scaffold. The crystal structure of compound 5 complexed with N9 neuraminidase and its activity against influenza neuraminidase suggested to us that a cyclopentane ring might be a suitable scaffold for a novel neuraminidase inhibitor. Compound 6 was designed to exploit the charged nature of the fourth pocket and was initially used for modeling cyclopentane neuraminidase inhibitors. This compound was synthesized as a racemic mixture, and its crystal structure bound to N9 neuraminidase revealed that the guanidino group occupies the fourth binding pocket replacing the existing water molecule and is involved in charge-based interactions with residues Asp 151, Glu 119, and Glu 227. This mode of binding for the guanidino group is analogous to the one observed in the crystal structure of zanamivir with influenza A neuraminidase.[6]

On the basis of the crystal structure of compound 6 with neuraminidase, structure 7 was modeled to exploit the small hydrophobic surface in the neuraminidase active site formed by Ala 246, Ile 222, and the aliphatic part of the side chain of Arg 224. The *n*-butyl side chain was designed to make hydrophobic contacts with these residues. The compound has asymmetric centers at the C1, C3, C4, and C1′ atoms of the cyclopentyl ring. In the initial synthesis, no efforts were made to fix the stereochemistry at the substituent positions of the ring. The only constraint during synthesis of compound 7 was to fix the guanidino group to be trans to the 1-acetylaminopentyl group. This lack of constraint led to a mixture of four racemates in the final compound. Neuraminidase crystals were used to select the most active isomer from this mixture. This was done by soaking a crystal of influenza neuraminidase in a solution of the isomers for 1 day and then collecting X-ray diffraction data from the crystal at a resolution of 2.5−2.0 Å. The resultant difference electron density in the active site of neuraminidase displayed unambiguously the stereochemistry of the active isomer. The structure of the bound isomer in the active site revealed that the carboxylic acid and 1-acetylaminopentyl group are trans to each other while the guanidino and carboxylic acid groups are cis to each other. This is not the expected stereochemistry of the active isomer based on the crystal structure of compound 6 with N9 neuraminidase where the carboxylic acid and acetylaminomethyl groups are cis to each other while the guanidino and carboxylic acid groups are trans to each other. The absolute configuration at C4 having the guanidino group is *R* in compound 7 and *S* in compound 6 as observed from the crystal structures of the complexes with the N9 enzyme. As a consequence of these differences in the stereochemistry, the guanidino group of compound 7 is oriented differently into the fourth pocket compared to the guanidino group of compound 6 and zanamivir.

Structural Comparison of Complexes of Compound 7 Bound to Influenza A and B Neuraminidases. X-ray crystallographic studies of compound 7 bound to influenza neuraminidase show that the same isomer is bound in both influenza A and B neuraminidase active sites. Interestingly, the *n*-butyl side chain of compound 7 adopts two different binding modes in the two structures (Figure 3). The *n*-butyl side chain in the influenza B neuraminidase active site is positioned against a hydrophobic surface formed by Ala 246, Ile 222, and Arg 224. However, in influenza A neuraminidase active site, the *n*-butyl side chain occupies a region

Figure 3. Superposition of compound **7** bound to influenza A N9 (green) and influenza B/Lec (yellow) neuraminidases. The protein side chains are from influenza A neuraminidase N9 active site.

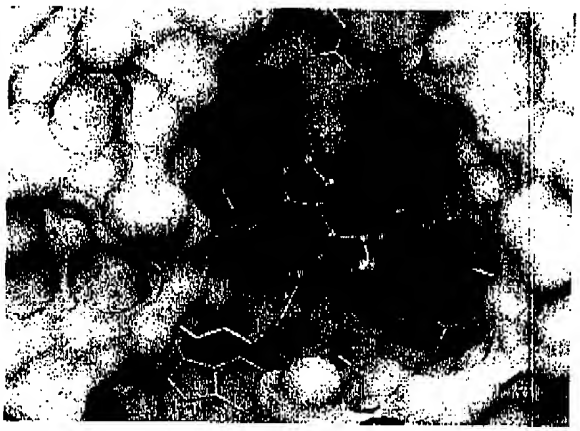


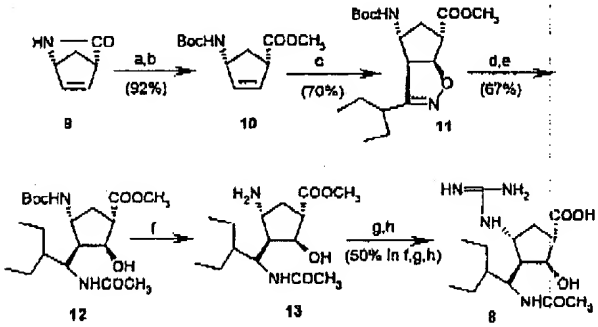
Figure 4. Binding of **8** in influenza A neuraminidase N9 active site. Small spheres are water molecules in the active site. Side chains interacting with **8** are displayed, while rest of the protein is represented as a surface.

formed by the reorientation of the side chain of Glu 276. In its new position, Glu 276 forms hydrogen bonds with the guanidino group of Arg 224. A similar rearrangement of Glu 276 is reported in the crystal structures of GS4071 and the carboxamide analogues of zanamivir bound to neuraminidase.[7,13] In addition, the crystal structure of compound **7** bound to influenza A neuraminidase shows some rearrangement of water structure in the acetamido binding pocket involving interactions with the NH of *N*-acetyl group.

The differences in the conformation of the *n*-butyl side chain in influenza A and B neuraminidase active sites could be due to small but critical differences in this highly conserved active site. It is possible that the rearrangement of Glu 276 is energetically less favorable in influenza B neuraminidase than in influenza A neuraminidase. It has been reported that the region surrounding Glu 276 is hydrophobic in influenza B but hydrophilic in influenza A.[13] The hydrophobic environment of Glu 276 in influenza B neuraminidase might be preventing the rearrangement of the Glu side chain, forcing the *n*-butyl moiety to occupy the already existing hydrophobic surface. Differences in the binding of inhibitors to influenza A and B neuraminidases, though not as dramatic as here, have been reported previously.[13]

Design and Selection of 8. We designed structure **8** to take advantage of both the hydrophobic pockets in the active site. Again, this compound was synthesized as a racemic mixture, and the crystal structure of compound **8** bound to influenza A neuraminidase was used to identify the active isomer (Figure 4). The active isomer was then synthesized using a stereospecific synthesis (Scheme 1). The crystal structure showed that the active isomer was the same as that of compound **7**, and there was a similar altered orientation of the guanidino group in the fourth pocket compared to the guanidino group in zanamivir. The guanidino group displaces the existing water molecule in the fourth pocket and has interactions with the enzyme that are similar to that of compound **7**. These differences in the orientation of the guanidino group (Figure 5) and the resultant differences in the interactions with the residues in the active site have implications in the cross-

Scheme 1[a]



[a] (a) HCl, MeOH; (b) $(Boc)_2O$, Et_3N; (c) PhNCO, Et_3N, 2-ethyl-1-nitrobutane; (d) H_2, PtO_2, MeOH, HCl (100 psi); (e) Ac_2O, Et_3N; (f) HCl, ether; (g) pyrazolecarboxamide·HCl, $(i\text{-Pr})_2EtN$; (h) NaOH.

reactivity of the influenza viruses resistant to zanamivir since they are known to have mutations in this part of the active site. The significance of these differences in the orientation of the guanidino group in the fourth pocket of the NA active site is emphasized by the observation that **8** retains its inhibitory activity against the zanamivir-resistant Glu 119 Gly variant of influenza A neuraminidase.[14,15] This is in spite of the fact that both zanamivir and **8** have a guanidino group that occupies the fourth pocket of the NA active site and Glu 119 is part of this pocket (Figure 1). The 2′-ethyl group of the 1′-acetylamino-2′-ethylbutyl group points into the hydrophobic pocket created by the rearrangement of the Glu 276 side chain, while the other ethyl group points toward the hydrophobic surface formed by the hydrocarbon chains of Arg 224 and Ile 222 (Figure 4). The crystal structure identified the isomer to be (1*S*,2*S*,3*R*,4*R*,1′*S*)-3-(1′-acetylamino-2′-ethyl)butyl-4-[(aminoimino)-methyllamino-2-hydroxycyclopentane-1-carboxylic acid (**8**).

Synthesis of 8. Compound **8** was prepared according to the route shown in Scheme 1. The starting material, (−)-lactam (2-azabicyclo[2.2.1]hept-5-en-3-one; Aldrich) **9**, was hydrolyzed with methanolic HCl; the resultant amino ester on reaction with Boc anhydride produced compound **10**. Cycloaddition (3+2) of compound **10** with the nitrile oxide produced from 2-ethyl-1-nitrobutane,

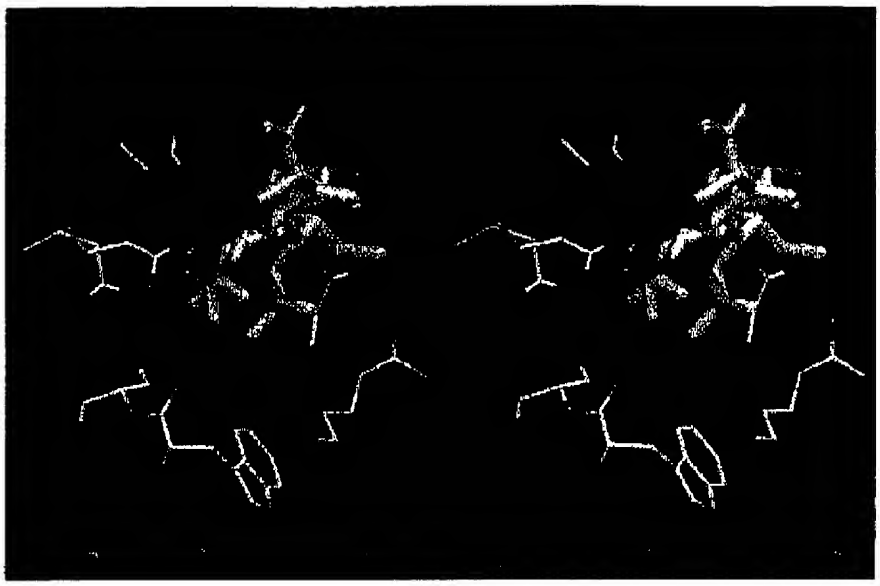


Figure 5. Superposition of **8** (orange) and **3** (blue) as they bound in influenza neuraminidase N9 active site to show the differences in the binding of the guanidino group and its interactions with the enzyme. The small red sphere is the water molecule that interacts with the nitrogen of the acetamido group of **8**. The crystal structure of zanamivir bound to influenza neuraminidase N9 was done at BioCryst Pharmaceuticals, Inc. and is similar to the published structure.

Table 1. IC_{50} Values for Influenza Neuraminidase Inhibition

compd	IC_{50}[a]	
	influenza A	influenza B
2	10 μM[b]	nd
3	0.3–2.3 nM[c]	1.5–17 nM[d]
4	0.01–2.2 nM[c]	6.4–24.3 nM[d]
5	40 μM[b]	nd
6	115 μM[b]	nd
7	0.1 μM[b]	nd
8	0.1–1.4 nM[c]	0.6–11 nM[d]

[a] IC_{50} values are the mean of 2–7 measurements; nd, not determined. [b] Measured using purified N9 neuraminidase enzyme. [c] The range of values represent measurements against 15 different strains of the influenza A virus. [d] The range of values represent measurements against 8 different strains of the influenza B virus.

phenyl isocyanate, and triethylamine gave cycloadduct **11** and other isomers (<10%). Cycloadduct **11** was isolated from the mixture of isomers and was hydrogenated in methanol with an equivalent amount of aqueous HCl in the presence of PtO_2 at 100 psi to give an amine hydrochloride, which was reacted with acetic anhydride to give the corresponding *N*-acetyl derivative **12**. Compound **12** on reaction with ethereal HCl gave deblocked amine **13**. Compound **13** on guanylation with pyrazole carboxamidine hydrochloride in DMF in the presence of diisopropylethylamine gave the corresponding guanidino ester, which on hydrolysis with NaOH gave the desired compound **8**.

In Vitro Neuraminidase Inhibition. Compound **8** was tested using a fluorimetric assay[16] for its ability to inhibit neuraminidase from several strains of influenza A and B viruses (Table 1). These data suggest that the in vitro potency of **8** is either comparable or superior to that of zanamivir and GS4071. The specificity of **8** for influenza neuraminidase was investigated by comparing its inhibitory activity with that for bacterial and mammalian neuraminidases and found to be at least 4 orders of magnitude less inhibitory of neuraminidases from these sources (data not shown). Taken together, the in vitro data show that **8** is a potent and highly specific inhibitor of influenza neuraminidase.

Table 2. Oral Efficacy of Compound **8** in Influenza Mouse Model

virus	dose, mg/kg/day b.i.d.	survival	mean day to death
A/Turkey/Mass/76 X A/Beijing/32/92 (H6N2)	1.0	9/9**	
	0.1	7/9*	9.0 ± 1.0
	0	1/9	8.8 ± 0.8

*P < 0.05, **P < 0.001 compared to dose = 0.

Oral Efficacy of Compound 8 in Mouse Influenza Model. Anesthetized mice (13–16 g) were infected intranasally with an approximate LD_{90} dose of the A/Turkey/Mass/76 X A/Beijing/32/92 (H6N2) influenza virus. Oral treatment with compound **8** (prepared in 0.5% CMC) was started 4 h prior to virus exposure and continued daily for 5 days. Nine mice were used in each of the drug and saline-treated groups, while five uninfected mice served as a normal control. Mice were examined daily for 21 days. Parameters for evaluation of antiviral activity included reduction in weight loss, prevention of death, and/or increase in mean day to death determined through 21 days. Table 2 shows that compound **8** was efficacious at a dose as low as 0.1 mg/kg/day b.i.d. (7/9 survived in the treated infected group while there was only one survivor in the untreated infected group).

Conclusions. We have discovered through structure-based drug design a novel, potent, selective, and orally active influenza neuraminidase inhibitor (**8**) that is structurally different from existing neuraminidase inhibitors. Protein crystallography was used to screen compounds containing mixtures of isomers in order to identify the active isomer. This is probably the first time where this technique has been used extensively in the

successful identification of a new enzyme inhibitor. We believe that this approach of analyzing ligands bound to protein crystals has wide applicability for screening mixtures of compounds to identify the active compound. Due to its in vitro and in vivo activity against influenza A and B, further studies with **8** in humans are warranted, and it is currently in human clinical trials for the management of influenza.

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Supporting Information Available: Experimental details about X-ray crystallographic data collection and processing. This material is available free of charge via the Internet at http://pubs.acs.org.

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